Recombinant *Phleum pratense* pollen allergen Phl p 4

Clues to new data for an old allergen?

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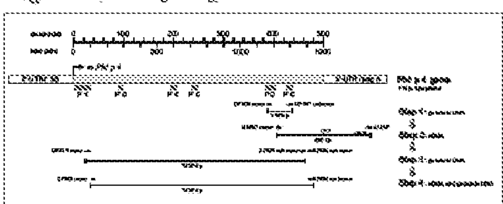
Introduction

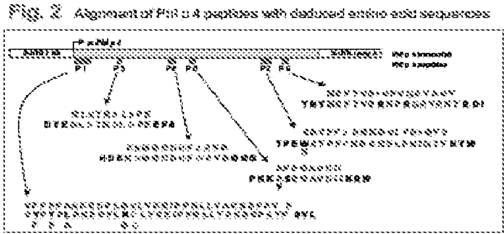
The group 4 allergens of grasses were first described more than 20 years ago and are well known as important major allergens of grass pollen allergy, one of the most common allergies world-wide. Phl p 4 is a basic glycoprotein that, together with Phl p 13, accounts for the high molecular weight fraction of grass pollen allergens. Frequencies of IgE sensitisation higher than 70% have often been reported (1-3), and therefore Phl p 4 seems to be as important as Phl p 5. Contrary to the situation for Phl p 5 and other important Phleum allergens, the primary structure of Phl p 4 has been discovered only recently, despite very considerable efforts in the past.

Fig. 1 Phl p 4 cloning strategy



Step 1: Degenerate oligonucleotide primer pools (DP830 and DP807) based on the Phl p 4 peptide sequences P3 and P6 have been used in a PCR reaction to amplify a small 140 bp internal DNA fragment of genomic *Phleum pratense* DNA.
Step 2: Based on that sequence a specific oligonucleotide primer (SP852) was designed to be used in a 3'-RACE PCR approach in combination with the anchor primer AUAP (Life technologies). A 489 bp fragment spanning the entire 3'-end of the Phl p 4 gene could be obtained.
Step 3: Two specific antisense primers (SP885 and SP890) have been designed on the basis of that sequence. These were used in combination with a degenerate sense primer pool (DP828) based on the N-terminal peptide P1 to amplify genomic Phl p 4 DNA in a "sense side mixed oligonucleotide, antisense side nested unique primer" PCR approach. A resulting 1320 bp fragment has been cloned and sequenced.
Step 4: A specific sense primer (SP863) has been designed and was used together with SP898 antisense to amplify a 1395 bp fragment of Phl p 4. Several independent PCR-products from genomic DNA as well as from cDNA have been sequenced to exclude PCR errors. Different variants of Phl p 4 could be detected. The identity of cDNA and genomic clones show that no intron sequences are present in the amplified region.

Fig. 2 Alignment of Phl p 4 peptides with deduced amino acid sequences

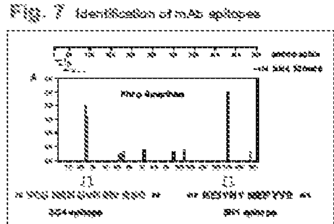


Natural Phl p 4 derived N-terminal and internal peptide sequences (first lines) aligned to confirm the deduced Phl p 4A (second lines) and Phl p 4B (third lines) genomic and cDNA sequences.

Results

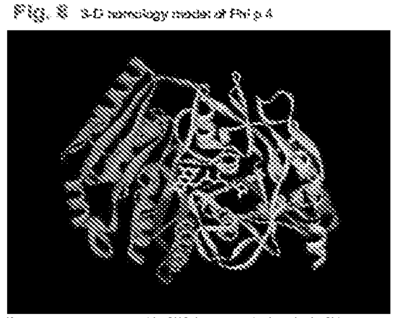
The experimental procedure that in the end led to the genomic and cDNA sequences of the gene based on a complex PCR strategy involving specific and degenerate primers (Fig. 1). The iden sequence has been confirmed to be Phl p 4 by alignment of the deduced amino acid sequence natural nPhl p 4 derived peptides (Fig. 2). The deduced amino acid sequences of two variants of m Phl p 4 consist of 500 amino acids each, with calculated molecular weights of 56 kDa and basic p 8,8 and 9,2, respectively. A sequence database homology search revealed similarities to berb bridge enzyme-like oxido-reductases (Fig. 3). Recombinant Phl p 4 was expressed in *E. co* inclusion bodies and has been subjected to a refolding procedure. However, the correct folding tu out to be difficult to achieve. Therefore we have expressed Phl p 4 in the methylotrophic yeast *P pastoris*. The *P. pastoris* derived Phl p 4 is highly soluble and has been purified via His-tag from cu supernatants. Purified recombinant Phl p 4 has been characterised by SDS-PAGE (Fig. 4), inhibition assay (Fig. 5), and protein dots using monoclonal antibodies, as well as IgE conta allergic subjects' sera (Fig. 6). The epitopes of two monoclonal antibodies 3C4, and 5H1 coul localised to the N-terminal and C-terminal domain, respectively (Fig. 7). A 3-D model of Phl p 4 generated on the basis of the vanillyl-alcohol oxidase (VAO) structure (Fig. 8).

Fig. 7 Identification of mAb epitopes



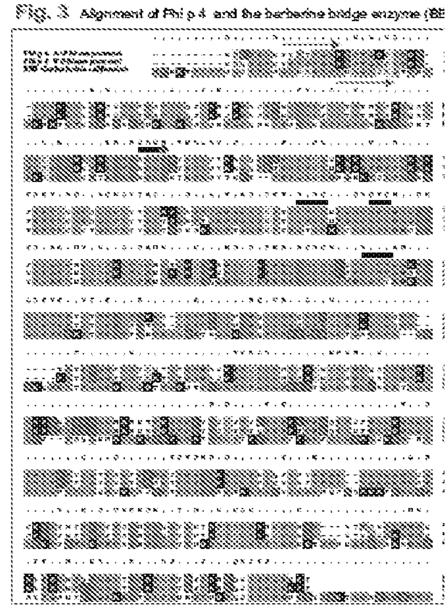
Overlapping 12mer peptides have been synthesized, biotinylated, and adsorbed to streptavidin coated MTP. The bound Phl p 4 specific monoclonal antibodies were detected by AP-α-mouse IgG.

Fig. 8 3-D homology model of Phl p 4



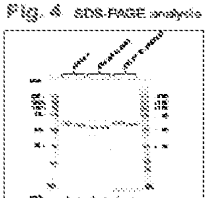
The consensus sequences of the BBE-like oxido-reductase family (3) have been used to align the Phl p 4 and vanillyl-alcohol oxidase (VAO) sequences. A 3-D homology model of Phl p 4 has been generated on the basis of the known structure of VAO (5) using the program Discovery Studio Modeling, Accelrys, St. Diego, U.S.A.

Fig. 3 Alignment of Phl p 4 and the berberine bridge enzyme (BB



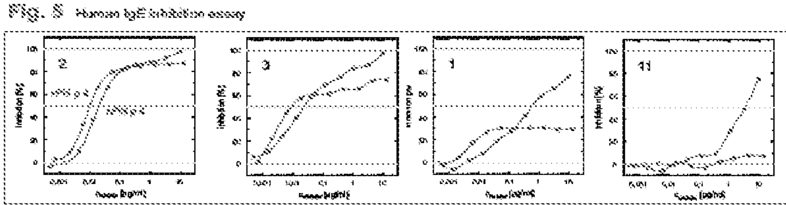
Sequence alignment of *Phleum pratense* Phl p 4 variant A, variant B and *Eschscholzia californica* berberine bridge enzyme. The conse [illegible]

Fig. 4 SDS-PAGE analysis



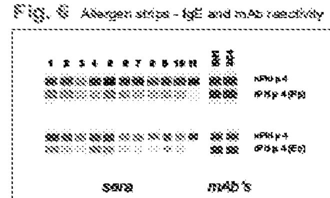
SDS-PAGE comparison of natural nPhl p 4, recombinant rPhl p 4 expressed in *E. coli*, and expressed in *P. pastoris*

Fig. 5 Human IgE inhibition assay



IgE inhibition assay using sera of grass pollen allergic individuals and purified natural Phl p 4 as solid phase. The inhibitory capacity of natural Phl p 4 (green dots) and recombinant Phl p 4 His purified from *P. pastoris* (red dots) supernatant cultures were compared. The sera of subjects 1, and 11 are known to contain significant amounts of cross reacting carbohydrate determinant (CCD) IgE antibodies.

Fig. 6 Allergen strips - IgE and mAb reactivity



Conclusion

The ability to produce recombinant Phl p 4 may represent a key step for the development of

References

1) R.E. Rossi et al. (2001), Allergy 56, 1180-1185